Bioconversion of Lithocholic Acid Under Anaerobic Conditions by Pseudomonas sp. Strain NCIB 10590

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The biotransformation of lithocholic acid by Pseudomonas sp. strain NCIB 10590 under anaerobic conditions was studied. The major products identified were androsta-1,4-diene-3,17-dione and 3-oxochol-4-ene-24-oic acid. The minor products included 17β-hydroxyandrostan-4-ene-3-one, 17β-hydroxyandrostan-1,4-diene-3-one, 3-oxo-Sβ-cholan-24-oic acid, 3-oxochol-1,4-diene-24-oic acid, 3-oxopregna-4-ene-20-carboxylic acid, and 3-oxopregna-1,4-diene-20-carboxylic acid. Anaerobiosis increases the number of metabolites produced by Pseudomonas sp. NCIB 10590 from lithocholic acid.

There have been several reports of lithocholic acid (LA) metabolism by bacteria (3, 4, 7, 8), but few which involve extensive degradation of the side chain.

Studies (4, 8) conducted under anaerobic conditions have shown that LA can be transformed by a wide range of bacterial species isolated from the rat intestine to give 3-oxo-Sβ-cholan-24-oic acid and 3β-hydroxy-Sβ-cholan-24-oic acid. Similar results were obtained by Borriello and Owen (3), who demonstrated that LA could be converted to 3-oxo-Sβ-cholan-24-oic acid by human mixed fecal bacteria and by Escherichia, Bacillus, Serratia, Chromobacterium, Proteus, Streptococcus, Erwinia, Enterobacter, and Klebsiella species.

Several studies have revealed that the metabolism of LA is more extensive under aerobic conditions. Hayakawa et al. (7) showed that LA can be converted to 3-oxochol-1,4-diene-24-oic acid by Arthrobacter simplex. Nagaosa et al. (9) demonstrated the production of androsta-1,4-diene-3,17-dione (ADD) from LA by A. simplex, whereas Tenneson et al. (18, 19) demonstrated the production of 3-oxopregna-1,4-diene-20-carboxylic acid and ADD from LA by Pseudomonas sp. strain NCIB 10590 (NCIB 10590).

Degradation of the steroid nucleus has also been observed when LA was incubated with A. simplex (7). In this case, a nonsteroidal product, [(R)-4-[4-α-(2-carboxyethyl)-3α-α-hydro-7αβ-methyl-5-oxindan-1β-yl]valeric acid, was isolated.

It was shown previously that NCIB 10590 has the ability to utilize a wide range of bile acids as the sole carbon source under aerobic conditions (15-19) and furthermore that resting cell suspensions of the same organism can utilize bile acids under anaerobic conditions (10-12, 14). Although a possible pathway of LA metabolism by bacteria was presented previously (18), many of the proposed intermediates had not been isolated and identified. The present investigation was conducted to ascertain whether or not NCIB 10590 was capable of degrading LA under anaerobic conditions. The only previous report of extensive LA metabolism under anaerobic conditions was described by Tenneson et al. (20), who showed that 3-oxopregna-1,4-diene-20-carboxylic acid and ADD could be produced by Escherichia coli.

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MATERIALS AND METHODS

Steroids and general reagents. LA was obtained from Roussel Uclaf (Paris), whereas 5α-cholestan-3β, androsta-1,4-diene-3,17-dione, 17β-hydroxyandrostan-4-ene-3-one, and 17β-hydroxyandrostan-1,4-diene-3-one were obtained from Koch Light Laboratories, Ltd. (Colnbrook, Buckinghamshire, England). General reagents, obtained from BDH (Poole, Dorset, England), were of AnalAR grade, and all solvents were redistilled before use.

Steroid analysis. Melting point, spectrophotometric, spectroscopic, thin-layer chromatographic (TLC), and gas-liquid chromatographic (GLC) determinations were carried out as described previously (10).

Organism. The organism used was NCIB 10590, identified as a nonpigmented, nonfluorescent Pseudomonas sp. group III-IV which has been deposited in the National Collection of Industrial Bacteria, Torrey Research Station, Aberdeen, United Kingdom.

Fermentation medium and incubation. The biotransformation medium used in this study consisted of (in grams per liter of distilled water): sodium lithocholate, 1.0; KH2PO4, 0.7; KH2PO4, 0.3; KNO3, 1.0; MgSO4 · 7H2O, 0.1; FeSO4 · 7H2O, 0.0025; ZnSO4 · 7H2O, 0.0025; and MnSO4 · 4H2O, 0.0025; final pH 7.2. The medium was dispensed as 1-litre portions into 2-litre Erlenmeyer flasks which were inoculated with 10% of a seed culture of NCIB 10590. The organism was grown to stationary phase at 28°C on an orbital shaker (LH Engineering, Stoke Poges, Buckinghamshire, England). Cells obtained by centrifugation from four 1-litre cultures were used to inoculate 4 liters of fresh culture medium. This gave a resting Pseudomonas cell suspension with a final cell density of 109 cells per ml. The culture medium was steamed for 30 min to remove dissolved oxygen and cooled immediately before inoculation. The top of the 4-liter fermentation bottle was loosened (to allow gaseous exchange) before incubation at 28°C for 6 weeks under 90% H2-10% CO2 (with palladium catalyst to remove residual oxygen), in an anaerobic churn. The anaerobic atmosphere of the churn was replenished at weekly intervals, substituting fresh catalyst at each stage. This technique was consistent with methodology for the growth of fastidious anaerobes of the human intestine (2).

Samples were also taken for optical density and pH measurement. Optical density (absorbance at 540 nm) measurements were made against a mineral salts blank on a Pye-Unicam SP.1800 spectrophotometer. The viability of the
culture was also checked at weekly intervals by streaking samples onto sodium lithocholate agar plates and incubating them aerobically at 28°C.

**Product analysis.** The progress of the fermentation was followed qualitatively by extraction of culture (cells plus medium) samples with an equal volume of ethyl acetate, which were subsequently checked by TLC and GLC for bacterial metabolites of LA.

The fermentation was terminated by direct extraction of the culture with ethyl acetate which, after removal of the solvent by evaporation and drying, gave 1.9 g of dry solids. The solid residue was dissolved in dichloromethane (15 ml) and separated by preparative TLC, yielding residual LA (710 mg) and eight fractions containing bacterial metabolites. Three neutral steroids, 2 (120 mg), 3 (40 mg), and 4 (30 mg), and five acidic steroids, 5 (72 mg), 6 (44 mg), 7 (60 mg), 8 (36 mg), and 9 (20 mg), were recrystallized from methanol-dichloromethane (1:9).

The neutral steroids (metabolites 2 through 4) were identical to authentic androsta-1,4-diene-3,17-dione, 17β-hydroxyandrost-4-ene-3-one, and 17β-hydroxyandrost-1,4-diene-3-one, respectively. Of the acidic steroids, metabolites 5 and 6 were identical to 3-oxochol-4-ene-24-oic acid and 3-oxochol-1,4-diene-24-oic acid, respectively, as described for the anaerobic degradation of hydoxycholeic acid (10). Metabolite 7 was identical to 3-oxopregna-1,4-diene-20-carboxylic acid, as described for the aerobic metabolism of LA (18). The two remaining acidic steroids, 7 and 9, gave the following analyses.

3-Oxopregna-1,4-diene-24-oic acid (7): Recrystallization of metabolite 7 gave white crystals (60 mg) at melting point 160°C (methyl ester). \( \lambda_{\text{max}} \), 241 nm; \( V_{\text{max}} \), 1.740 (22-COOCH\(_3\)), 1.670, and 1.620 cm\(^{-1}\) (4-ene-3-one); \( \delta \), 0.72, 1.17 (6H, s, 18-CH\(_3\)), and 19-CH\(_3\)), 1.19 (3H, s, 21-CH\(_3\)), 3.64 (3H, s, 22-OCH\(_3\)), and 5.71 (1H, s, 4-H). M\(^+\) 358 (76%) (C\(_{23}\)H\(_{34}\)O\(_3\)) requires M\(^+\) 358, m/e 124 (4-ene-3-one, 100%) and m/e 271 (M -87, 4C-side chain, 11%). GLC \( R_f \) 3.09, TLC \( R_f \) 1.19, after oxidation \( R_f \) 1.19, after acetylation \( R_f \) 1.19, and after reduction \( R_f \) 1.19. 3-Oxopregna-1,4-diene-20-carboxylic acid (9): Recrystallization of metabolite 9 gave white crystals (20 mg) at melting point 122 to 126°C. \( \lambda_{\text{max}} \), 286 nm (\( \epsilon \) 80); \( V_{\text{max}} \), (methyl ester) 1.740 (24-COOCH\(_3\)) and 1.710 cm\(^{-1}\) (3-ketone); \( \delta \), 0.68, 1.00 (6H, s, 18-CH\(_3\)), and 19-CH\(_3\)), 0.96 (3H, s, 21-CH\(_3\)), and 3.63 (3H, s, 24-OCH\(_3\)). M\(^+\) 388 (99%) (C\(_{23}\)H\(_{34}\)O\(_3\)) and m/e 278 (M\(^-\) -115, 6C-side chain, 100%). GLC \( R_f \) 3.92, TLC \( R_f \) 1.30, after oxidation \( R_f \) 1.30, after acetylation \( R_f \) 1.30, and after reduction \( R_f \) 0.92.

**RESULTS**

**LA transformation.** Resting cell suspensions of NCIB 10590 metabolized sodium lithocholate rather slowly over a 6-week period in a mineral salts medium. The stability of the culture and the course of bile acid transformation were followed by measurements of cell density and concentration of 1,4-diene-3-oxo steroids. Cell density remained relatively constant throughout the experiment, dropping to 0.9 (absorbance at 540 nm) from an initial value of 1.0 (absorbance at 540 nm), whereas culture, pH, and viability were constant throughout. The concentration of 1,4-dien-3-oxo steroids in the medium did not reach a maximum until 6 weeks of fermentation and required 1 week of incubation before the induction of steroid-degrading enzymes occurred.

NCIB 10590 degraded LA under anaerobic conditions and yielded eight metabolites (Table 1). The suggested structures are depicted in Fig. 1 and 2.

The three neutral metabolites (2 through 4) were identical to androsta-1,4-diene-3,17-dione, 17β-hydroxyandrost-4-ene-3-one, and 17β-hydroxyandrost-1,4-diene-3-one, respectively, in their spectroscopic and mass spectral properties.

The acidic fraction contained five metabolites. Metabolites 5 and 6 were identical to 3-oxochol-4-ene-24-oic acid and 3-oxochol-1,4-diene-24-oic acid, as described previously (10). Metabolite 7 was identical to 3-oxopregna-1,4-diene-20-carboxylic acid, described in the aerobic catabolism of LA (19). However, two of the acidic metabolites have not been described previously as aerobic or anaerobic catabolites of LA.

Metabolite 7 gave an intense ion (base peak) at m/e 124 (100%) in its mass spectrum typical of a steroidal 4-ene-3-one A-ring structure (10). Confirmation of the A-ring structure was provided by the UV spectrum (\( \lambda_{\text{max}} \) 242 nm, di-β-substituted αβ-unsaturated ketone, double-bond exocyclic) (12) and by the nuclear magnetic resonance spectrum (one vinylic proton at 5.71 ppm). Compound 7 could not be oxidized, acetylated, or reduced, suggesting the absence of both hydroxy and ketone groups. This was confirmed by the infrared spectrum. Metabolite 7 has therefore been assigned the structure 3-oxopregna-1,4-diene-20-carboxylic acid. The methyl ester of metabolite 9 gave a mass spectrum very similar to that of methyl-3-oxo-5β-cholanoate described by Eneroth et al. (6) as a fecal component and contained the

![FIG. 1. Neutral metabolites isolated after the anaerobic degradation of LA by NCIB 10590.](http://aem.asm.org)
anaerobic conditions there is also significant accumulation of acidic metabolites. In this respect, LA biotransformation differs from that of other bile acids because during the catabolism of deoxycholic acid (1, 14), chenodeoxycholic acid (12, 15), cholic acid (11, 16), and hyodeoxycholic acid (10, 17) by NCIB 10590 substantial accumulation of acidic steroids occurs under both aerobic and anaerobic conditions. It is probable that the nuclear hydroxyl functions present on the latter bile acids inhibit side-chain cleavage. This is best illustrated by the catabolism of deoxycholic acid (1, 14). For side-chain cleavage of deoxycholic acid to occur, the 12α-hydroxyl group has to be epimerized to a 12β-hydroxyl group (1). This causes appreciable accumulation of acidic steroids in the culture medium.

Aerobic catabolism of LA by NCIB 10590 (19) shows a close resemblance to cholesterol metabolism by the same organism (21). During cholesterol metabolism, specific enzyme inhibitors, such as α,α′-dipyridyl and n-propanol, must be included in the medium for accumulation of both acidic and neutral steroid metabolites. Although enzyme inhibitors are not required for the accumulation of metabolites in aerobic LA metabolism, the metabolites are dominated by neutral steroids. It is apparent that the lack of nuclear hydroxyl groups on the acidic metabolites of LA and cholesterol enhances side-chain cleavage.

The present study shows that, under anaerobic conditions, the lack of molecular oxygen for 9α-hydroxylation of the steroid nucleus is sufficient to cause inhibition of side-chain cleavage as evidenced by the detection of metabolites 5 through 9 in the culture medium. Nevertheless, it is evident that NCIB 10590 is capable of degrading the bile acid nucleus under anaerobic conditions because more than 50% of the starting material could not be accounted for (Table 1). A mechanism for nuclear degradation of 12α-hydroxylated C19 compounds under anaerobic conditions has already been proposed (14). However, it is difficult to envisage nuclear ring fission of molecules lacking hydroxy groups in the absence of molecular oxygen. This phenomenon requires further study.

However, it is evident (Fig. 3) that the metabolism of LA under anaerobic conditions by NCIB 10590 proceeds in a manner similar to that for other bile acids. To summarize, LA is initially oxidized to 3-oxo-5β-cholan-24-oic acid (metabolite 9) followed by nuclear steroid oxidation yielding 3-oxochol-4-ene-24-oic acid (metabolite 5) which in turn can be oxidized at C1-C3 to give 3-oxochol-1,4-diene-24-oic acid (metabolite 6). Thereafter, the A-ring, unsaturated C32 intermediates are metabolized sequentially by β-oxidation via C22 acidic intermediates (metabolites 7 and 8) to C19 neutral products (metabolites 2 through 4), as previously proposed (10).

In conclusion, although NCIB 10590 is capable of degrading LA to a wider range of products under anaerobic conditions than under aerobic ones, the total yield is over 50% lower, whereas the time taken is significantly longer. Nevertheless, this study has enabled the isolation of several additional metabolites not previously recorded as products of LA metabolism. A possible pathway of LA metabolism by NCIB 10590 under anaerobic conditions is depicted in Fig. 3.

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LITERATURE CITED


